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Isolation, Characterization, and Mapping of Tn*5* Insertions into the 140-Megadalton Invasion Plasmid Defective in the Mouse Sereny Test in *Shigella flexneri* 2a

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Using *Shigella flexneri* 2a YSH6000, we isolated 304 independent Tn*5* insertion mutants in the 230-kilobase invasion plasmid, pMYSH6000. The site of each Tn*5* insertion was assigned to 23 *Sal*I fragments on the previously made *Sal*I cleavage map of pMYSH6000. Among the 304 insertions, 150 were negative in expression of four phenotypes examined (mouse Sereny test [Ser], invasion into epithelial cells [Inv], Congo red binding [Pcr], and inhibition of bacterial growth [Igr]): 12 were Ser$^-$ Inv$^+$ Pcr$^+$ Igr$^+$, and 142 were positive in all four phenotypes. Tn*5* insertions in the avirulent mutants were distributed in two separate *Sal*I fragments, F and G, and in four contiguous *Sal*I fragments, B, P, H, and D. Fragment G contains a novel class of determinant(s) which is required only for Ser$^+$ but not for Inv$^+$, Pcr$^+$, and Igr$^+$. Fragment F contains the previously characterized *virF* locus. B, P, H, and D each contained both virulent and avirulent Tn*5* insertions. This indicates that more than two gene clusters exist within this region. Both are required for expression of all four virulence phenotypes.

The genetic determinants required for virulence of shigellae are located on both the chromosome (10) and a 100- to 140-megadalton plasmid (11). On the chromosome of *Shigella flexneri*, at least three separate regions have been associated with virulence. Invasion by shigellae of colonic epithelia and their multiplication therein are the primary requirements for the virulence leading to bacillary dysentery (2). Presence of the large virulence plasmid is essential for these events.

It has been known that the virulence of *S. flexneri* is an unstable property which is frequently lost upon refrigeration (5) or subculturing (13). Avirulent derivatives have usually lost three virulence-associated phenotypes: (i) Sereny test (Ser) (6, 14); (ii) epithelial invasion of in-vitro-cultured cells (Inv) (2); (iii) Congo red binding ability (Pcr) (1, 5, 8, 9, 13). Such avirulent mutants frequently contain a virulence plasmid with molecular alterations (1, 5, 13). This correlation and the ease with which avirulent mutants are isolated has been attributed to the in vitro growth rate of the mutants being faster than that of wild-type organisms (a phenotype designated as Igr for inhibition of bacterial growth) (13).

In previous work (13), we isolated 40 avirulent derivatives of *S. flexneri* 2a YSH6000 and analyzed them for molecular alterations of their plasmid. All of these were Ser$^-$ Inv$^-$ Pcr$^-$ Igr$^-$. We also made a gene library of fragments generated by *Sal*I endonuclease treatment. Based on these data, a circular *Sal*I restriction map was constructed. These studies identified two virulence-associated sites on the plasmid about 50 kilobases (kb) apart (13). One of them, tentatively called *virF*, has been cloned and characterized (9).

Recently, Maurelli et al. (4) cloned a 37-kb DNA cluster from the 140-megadalton plasmid of *S. flexneri* 5 into a cosmid vector. Their clone was sufficient for providing the invasive phenotype when introduced into a plasmid-free rough *Shigella* host. However, it provoked neither the Sereny reaction nor plaque formation in the tissue monolayer cell assay described by Oaks et al. (7). They also identified at least three genes on this cloned fragment.

In the present study of 304 independent Tn*5* insertion mutants, several additional virulence-associated regions were identified. One of these is essential solely for the Ser$^+$ phenotype, whereas those remaining are needed for all four phenotypes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and chemicals. LN (12), Penassay (Difco Laboratories, Detroit, Mich.), and Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) broths and their solidified agars were used. Congo red was purchased from Sigma Chemical Co., St. Louis, Mo.

Isolation of deletion derivatives of pMYSH6000. Fully virulent isolates of YSH6000 were plated on LN agar and incubated at 37°C for 20 h. Numerous Pcr$^+$ single colonies were separately inoculated into 2 ml of LN broth and grown fully at 37°C. Each culture was diluted 10^{-7}-fold with LN broth and repeatedly subcultured twice daily for several days. At this time, each culture was plated for isolated colonies on LN agar. One single, large (Igr$^-$) colony, usually carrying a detectable molecular change in the 230-kb plasmid, was taken from each subculturing lot and characterized.

Insertion mutagenesis of pMYSH6000. A thermosensitive replication mutant of R388 with Tn*5* (manuscript in preparation) was conjugally introduced into YSH6000 by selecting for trimethoprim (Tp) resistance. A Tp^r kanamycin-resistant (Km^r) transconjugant was spread on an LN agar plate to yield about 1,000 separate colonies at 30°C. Each of these isolates was grown at 30°C in LN broth containing 50 μg of kanamycin per ml, spread on agar containing kanamycin, and then grown at 42°C. At this time, Km^r Tp^s clones were isolated from each colony. Plasmid DNA was extracted, digested with *Sal*I, and resolved in 0.9% agarose by gel electrophoresis. The Tn*5* transposon produces two frag-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Virulence in mouse Sereny test	Source or reference
YSH6000	Virulent strain of *S. flexneri* 2a	+	13
YSH6104	YSH6000 carrying a deletion derivative of pMYSH6000 (del-53; deletion of part of fragment J through half of fragment C)	−	13
YSH6106	YSH6000 carrying a deletion derivative of pMYSH6000 (del-23; deletion of part of fragment J through part of fragment F)	−	13
YSH6107	YSH6000 carrying a deletion derivative of pMYSH6000 (del-37; deletion of part of fragment G through part of fragment T)	−	13
YSH6109	YSH6000 carrying a deletion derivative of pMYSH6000 (del-18; deletion of part of fragment L and part of fragment F)	−	13
YSH6201	YSH6000 carrying R388 *rep* (Ts)::Tn*5*	+	This study
YSH6202-M94	YSH6000 carrying a Tn*5* insertion derivative of pMYSH6000 (G::Tn*5*)	−	This study
YSH6203-S84	YSH6000 carrying a Tn*5* insertion derivative of pMYSH6000 (B::Tn*5*)	−	This study
YSH6204-S296	YSH6000 carrying a Tn*5* insertion derivative of pMYSH6000 (P::Tn*5*)	−	This study
YSH6205-M5	YSH6000 carrying a Tn*5* insertion derivative of pNYSH6000 (H::Tn*5*)	−	This study
YSH6206-S325	YSH6000 carrying a Tn*5* insertion derivative of pMYSH6000 (D::Tn*5*)	−	This study
YSH6207-S74	YSH6000 carrying a Tn*5* insertion derivative of pMYSH6000 (F::Tn*5*)	−	This study
pMYSH6000	230-kb virulence plasmid of YSH6000		13
R388 *rep*(Ts)::Tn*5*	Thermosensitive replication mutant of R388::Tn*5*		In preparation

ments of 2.4 and 3.3 kb when cut with *Sal*I. Thus, if Tn*5* is inserted into any of the 23 *Sal*I fragments of pMYSH6000, these fragments disappear and two new fragments produced by fusion of each of the cleaved fragments of the lost *Sal*I fragment to either a 2.4- or a 3.3-kb Tn*5* fragment appear. A total of 304 Tn*5* insertions were defined in this manner.

Isolation and characterization of plasmid DNA. Large- and small-scale preparation of plasmid DNA has been previously described (13). Restriction endonucleases were used as described by Maniatis et al. (3). Electrophoresis of restriction endonuclease-cleaved DNA was performed in horizontal 0.9% agarose slab gels in a Tris-acetate buffer system (160 mM Tris base, 80 mM sodium acetate, 8 mM EDTA, pH 8.3) (12) at 70 mA for 18 h. To detect uncleaved plasmid bands, we reduced the concentration of the Tris-acetate buffer to one-fourth the original ionic strength.

Mouse Sereny test. Male ICR mice at 4 weeks of age were used for the mouse Sereny test (6). A single bacterial colony to be tested was inoculated into 5 ml of Penassay broth and grown at 37°C overnight with shaking. Cells were centrifuged, washed once with saline, and suspended in 0.2 ml of saline which contained roughly 10^{11} cells per ml. Concentrated cell suspension (30 μl) was deposited into the conjunctival sac of one eye, and the other eye was used as a negative control. Usually, two mice were used for each test, and the results were judged after 24 h.

Invasion of tissue culture monolayers. Essentially the method described by Maurelli et al. (5) was used to test invasive ability. Rhesus monkey epithelial cell line LLC-MK (15), kindly supplied by A. Nakamura of National Institute of Health, Tokyo, Japan, was used in our study.

Congo red binding ability. Congo red binding ability was tested by growing bacteria at 37°C on Congo red-containing Trypticase soy agar (8).

Test for inhibition of bacterial growth. Fresh LN broth cultures of the test bacteria grown at 37°C were plated on Trypticase soy agar so as to yield separate colonies after growth at 37°C for 20 h. Scoring was done by comparing the sizes of colonies with those of the virulent wild type. This simple method was found to be reliable by comparing the sizes of colonies and their previously reported (13) generation times.

RESULTS

From the analysis of 40 deletion or IS*1* insertion mutants isolated in a previous study (13), two virulence-associated sites were identified: a small deletion extending over *Sal*I fragments F and L and an IS*1* insertion into *Sal*I fragment H, positioned about 50 kb apart. Both genetic alterations result in an Ser^- Inv^- Pcr^- Igr^- phenotype. A determinant, tentatively designated as *virF*, was identified within the gene region encompassed by this deletion (13). To determine whether there are any other virulence-associated regions on this plasmid, we isolated and characterized additional virulence-defective derivatives. Molecular alterations of 73 mutant plasmids, including the 40 previously analyzed (13), are localized on the *Sal*I restriction map (data not shown). Previous conclusions about the existence of deletion hot spots were confirmed. No additional virulence determinants were identified, however. It seems noteworthy that the distribution of the deletions among 23 *Sal*I restriction fragments appears uneven. Fragment C and its adjacent region are among the least affected by deletion. This appears to reflect that the right half of fragment C in the *Sal*I restriction map of pMYSH6000 contains an essential region for replication (manuscript in preparation).

Isolation and characterization of Tn*5* insertions of pMYSH6000. Using a thermosensitive replication mutant of R388 carrying Tn*5*, we isolated about 1,000 independent Tn*5* insertion mutants of YSH6000. In Fig. 1, representative electrophoretic patterns of *Sal*I-cleaved pMYSH6000 fragments of such mutants are shown. The sites of Tn*5* insertion are listed in Table 2, together with the length of each *Sal*I fragment and the number of Tn*5* insertions assigned to it. In the fourth column, a ratio of the number of Tn*5* insertions per kilobase of DNA is shown. The results of Sereny and Congo red binding tests of 304 Tn*5* insertions are listed in Table 2. Tn*5* insertions into *Sal*I fragments A, C, E, I, J, K, L, M, N, O, Q′, R, S, and T produced no change in virulence phenotypes and presumably contain no virulence determinants. Some Tn*5* insertions into *Sal*I fragments B, D, F, H, and P resulted in the loss of Ser^+ and Pcr^+ phenotypes. Among them was the virulence determinant on *Sal*I fragment F, which has already been cloned and characterized (9) as a

1.0-kb virulence determinant, *virF*. This determinant is sufficient to confer the Pcr$^+$ phenotype upon *Escherichia coli* K-12 and is essential but not sufficient for virulence of the smooth strain of *S. flexneri* 2a, YSH6000. A total of 150 Ser$^-$ and Pcr$^-$ mutants were Inv$^-$ (Fig. 2B). All 12 Ser$^-$ Tn*5* insertions into *Sal*I fragment G were Inv$^+$, however, in the same test (Fig. 2A). Thus, these 150 mutants had lost three virulence-associated phenotypes. Ser, Inv, and Pcr. In contrast, 12 of 17 Tn*5* insertions into *Sal*I fragment G were Ser$^-$ but Inv$^+$ (Fig. 2) and Pcr$^+$.

Growth rate of Tn*5* insertion mutants. In a previous study (13), all Pcr$^-$ mutants of YSH6000 were found to have shorter generation times in artificial media at 37°C than did the virulent wild type. As expected, all insertion mutants with Ser$^-$, Inv$^-$, and Pcr$^-$ phenotypes had this property (Igr$^-$), whereas all of the Ser$^-$, Inv$^+$, and Pcr$^+$ insertion mutants within *Sal*I fragment G retained the Igr$^+$ phenotype (data not shown).

***virF* is the only virulence determinant located between *Sal*I fragments J and C.** When a *virF*-containing DNA fragment of about 1.2 kb cleaved from *Sal*I fragment F (9) was cloned and transformed to four smooth strains derived from YSH6000, del-18 (YSH6109), del-23 (YSH6106), and del-53 (YSH6104), but not del-37 (YSH6107) (Table 1; Fig. 4 of reference 13), became virulent. This observation further confirmed our previous conclusion that *virF* is essential but not sufficient for virulence of YSH6000 (9). It also showed that no virulence determinant except *virF* exists within a range of about 90 kb from *Sal*I fragment J through half of *Sal*I fragment C (Fig. 3).

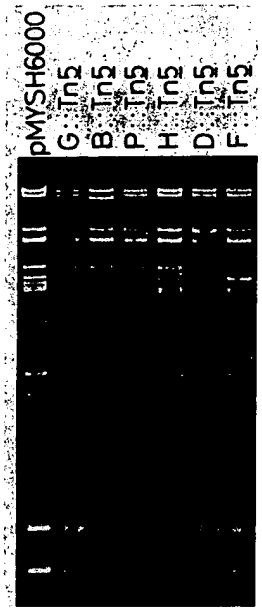

FIG. 1. *Sal*I restriction cleavage patterns of representative avirulent Tn*5* insertion plasmids of pMYSH6000. Sites of Tn*5* insertion are indicated at the tops of the lanes. Tn*5*-inserted pMYSH6000-derivative plasmids (left to right) were extracted from YSH6202-M94, YSH6203-S84, YSH6204-S296, YSH6205-M5, YSH6206-S325, and YSH6207-S74 (Table 1). The leftmost lane shows the *Sal*I restriction pattern of the parental virulence plasmid, pMYSH6000.

DISCUSSION

Two virulence determinants located about 50 kb apart had been previously identified on the virulence plasmid of *S. flexneri* 2a (13). One of these, designated as *virF*, is associated with the Pcr$^+$ phenotype and is essential for virulence. *virF*, as shown in this study, complements deletion mutant del-53 (YSH6104) (Table 1; Fig. 4 of reference 13). This finding indicated that no virulence determinant except *virF* exists within 90 kb between *Sal*I fragment J through half of *Sal*I fragment C (Fig. 3).

To identify additional virulence determinants on pMYSH6000, we isolated 304 independent Tn*5* insertion mutants of the plasmid. Of these, 142 were Ser$^+$ and 156 were Ser$^-$. The ratio of the number of Tn*5* insertions per

TABLE 2. Distribution of Tn*5* insertions in *Sal*I restriction fragments of pMYSH6000 and their virulence-associated phenotypes

*Sal*I restriction fragment	Length (kb)	No. of Tn*5* insertions obtained	No. of Tn*5* insertions/kb	No. of isolates with the phenotype:		
				Ser$^+$ Inv$^+$ Pcr$^+$ Igr$^+$	Ser$^-$ Inv$^-$ Pcr$^-$ Igr$^-$	Ser$^-$ Inv$^+$ Pcr$^+$ Igr$^+$
A	43.1	40	0.93	40	0	0
B	32.6	28	0.86	22	6	0
C	16.9	4	0.24	4	0	0
D	15.8	63	3.99	7	56	0
E	15.7	10	0.64	10	0	0
F	11.2	14	1.25	4	10	0
G	10.3	17	1.65	5	0	12
H	9.9	72	7.27	1	71	0
I	9.6	13	1.35	13	0	0
J	9.4	6	0.64	6	0	0
K	7.8	9	1.15	9	0	0
L	7.5	4	0.53	4	0	0
M	6.6	6	0.91	6	0	0
N or O	5.7, 5.7	1	0.18	1	0	0
P	5.1	10	1.96	3	7	0
Q'	4.3	6	1.40	6	0	0
Q	3.5	0	0	0	0	0
R'	2.4	0	0	0	0	0
R	2.2	1	0.45	1	0	0
S'	1.8	0	0	0	0	0
S	1.6	0	0	0	0	0
T	1.5	0	0	0	0	0

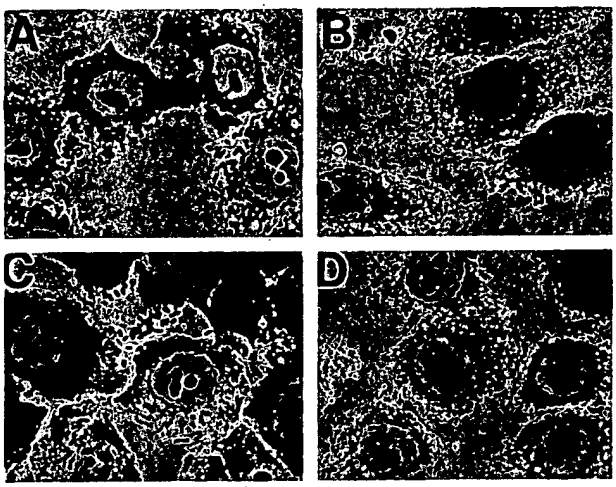

FIG. 2. LLC-MK epithelial cell monolayers infected with representative avirulent mutants of YSH6000. Panels: (A) YSH6202-M94(pMYSH6000*Sal*I fragment G::Tn*5*) (Ser$^-$ Inv$^+$ Pcr$^+$ Igr$^+$); (B) YSH6207-S74(pMYSH6000*Sal*I fragment F::Tn*5*) (Ser$^-$ Inv$^-$ Pcr$^-$ Igr$^-$); (C) YSH6000(pMYSH6000) (Ser$^+$ Inv$^+$ Pcr$^+$ Igr$^+$); (D) YSH6100(pMYSH6000del-17) (Sel$^-$ Inv$^-$ Pcr$^-$ Igr$^-$).

kilobase of DNA of all the avirulent Tn*5* insertions was larger than that of the virulent derivatives (Table 2), implying that during isolation of the mutants Igr$^-$ insertion mutants may have overgrown and hence be isolated at a frequency higher than that calculated from the length of the *Sal*I fragment. Of 162 Ser$^-$ insertions, 150 were also Inv$^-$ Pcr$^-$ Igr$^-$. The remaining 12 were Ser$^-$ Inv$^+$ Pcr$^+$ Igr$^+$. It is highly likely that three phenotypes, Inv$^+$, Pcr$^+$, and Igr$^+$, are pleiotropic and encoded by common genetic determinants because all virulence determinants can be divided phenotypically into two groups: one required for all four phenotypes and another required for Ser$^+$ only.

The Tn*5* insertions which affected expression of all four

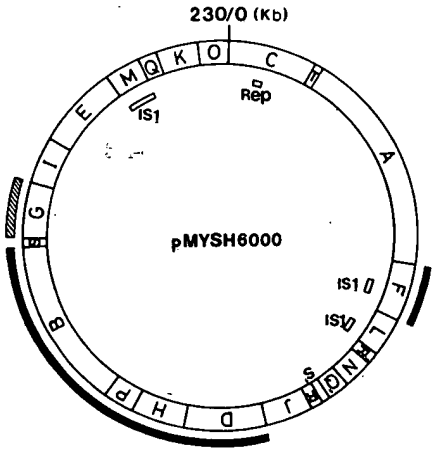

FIG. 3. Circular *Sal*I restriction map pMYSH6000. The outermost, solid segments (▬) indicate the *Sal*I fragments containing determinants responsible for Ser$^+$, Inv$^+$, Pcr$^+$, and Igr$^+$, and the hatched segment (▨) indicates those responsible only for Ser$^+$. The open boxes inside the map in the *Sal*I fragments F, L, and M indicate the IS*1*-like elements previously identified (13), and in *Sal*I fragment C is the region required for plasmid maintenance (manuscript in preparation).

phenotypes are located in *Sal*I fragments B, P, H, D, and F (Fig. 3). Fragment F contains *virF* (9), whereas the remaining four fragments are contiguous and comprise about 63 kb in total. One of them, fragment H, overlaps with the IS*1* insertion, ins-33, described previously (13). Since Tn*5* insertion into any of the four fragments produced both virulent and avirulent mutants, there appear to be at least two clusters of genes within this region. This is the minimum number expected from the data if one gene region straddles fragments B and P and another straddles fragments H and D (Fig. 3). According to our preliminary analysis, the determinant(s) in fragment B is within 6 kb of fragment P, and that in fragment D is within 12 kb of fragment H. Thus, the size of the cluster is estimated to be less than 33 kb.

There are several differences between the results of Maurelli et al. (4) and our findings. Their studies of a 37-kb fragment from the 140-megadalton plasmid, pWR100, of *S. flexneri* 5 revealed that this region is sufficient for invasion (4). In contrast, our studies of plasmid pMYSH6000 revealed a cluster of less than 33 kb representing four contiguous *Sal*I fragments which are required, but not sufficient, for invasion. The determinant *virF* is required for invasion and is located more than 30 kb apart from this cluster. In plasmid pMYSH6000, there appears to be one additional determinant on *Sal*I fragment G. The distance between fragment G and the junction of fragments H and D is greater than 37 kb. These differences in findings may reflect only the difference of the strain and may be ascribed to divergency of the 140-megadalton plasmids in *S. flexneri*. We are currently trying to determine the detailed regions associated with virulence conferred by the large plasmid.

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